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Chromatin clearance in C57Bl/10 mice: interaction with heparan sulphate proteoglycans and receptors on Kupffer cells

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SUMMARY

Chromatin is an important autoantigen in the pathogenesis of systemic lupus erythematosus (SLE) as an immunogen and as a part of nephritogenic immune complexes. Earlier studies focused on clearance of DNA. However, DNA released into the circulation from dying cells is found associated with histones in nucleosomes. The liver is the major organ involved in clearance of chromatin from the circulation of mice. Heparan sulphate proteoglycans (HSPG) have been implicated in the clearance of various charged molecules. Receptor-mediated clearance of ssDNA by the liver has also been reported. Because chromatin contains positively charged histones in addition to DNA, we wished to determine if HSPG and/or DNA receptors are involved in chromatin clearance. The rate of clearance of H1-stripped chromatin from the bloodstream of C57Bl/10 mice was markedly decreased by prior treatment of mice with Heparinase I. Clearance was also inhibited by heparin, heparan sulphate, and DNA, but not by colominic acid. DNA was the most effective inhibitor of clearance and released chromatin from sites of clearance. Depletion of Kupffer cells and splenic macrophages using liposome-encapsulated Clodronate (dichloromethylene bisphosphonate) markedly inhibited chromatin clearance. These data suggest that chromatin clearance is mediated by charge interactions with cell surface HSPG and by DNA receptors. Clearance and degradation of chromatin require functional macrophages in the liver and spleen.

Keywords *in vivo* animal models lupus autoimmunity chromatin Kupffer cells

INTRODUCTION

Chromatin is considered to be one of the primary targets of autoantibodies in patients with systemic lupus erythematosus (SLE). Autoantibodies to chromatin and to DNA result in the formation of immune complexes in these patients. These immune complexes are responsible for the systemic disease manifestations and are particularly important in the development of glomerulonephritis. Early clearance studies focused on DNA, which is cleared very rapidly from the circulation [1–5]. However, studies of DNA in the circulation have demonstrated that the form of DNA is a repeat of 200–800 base pairs complexed with histones [6]. Chromatin may be released from cells injured by necrosis or by apoptosis. In apoptosis, the chromatin is cleaved into nucleosome repeats giving the characteristic ladder pattern of DNA fragments on gel electrophoresis. Thus, chromatin, in the form of oligonucleosomes, is the probable configuration of circulating DNA. The mechanism of clearance of chromatin from the circulation is therefore of interest.

We first reported on the clearance of chromatin from the circulation of mice [7,8]. The initial studies were done with core particles, which represent the limit digest of chromatin treated with micrococcal nuclease. Those studies showed that core particles were cleared rapidly and were deposited in the liver. Clearance was blocked by preinjection of excess chromatin or by DNA. Gauthier *et al*. also studied the clearance of core particles and determined that the clearance of core particles is saturable [9]. More recently, we compared the clearance of core particles and H1-stripped chromatin, which is a soluble form of chromatin composed of oligomers of nucleosomes [8]. The rate of clearance was more rapid for H1-stripped chromatin than for core particles and was strongly influenced by the presence of the pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP).

Chromatin and core particles are both cleared by the liver [7,8]. Both chromatin and core particles contain highly charged, basic amino acids. Previous studies suggested that the clearance of core particles was blocked by succinylated albumin, ssDNA [9],

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dsDNA, and chromatin [8]. However, whether these inhibitors were preventing charge interactions or more specific binding to receptors was not determined.

In the present study, we examine the clearance from the blood of H1-stripped chromatin of relatively low molecular weight. This is most consistent with the size of chromatin reported in the circulation presumably as the result of cell breakdown by apoptosis or necrosis [6]. We examine the ability of DNA, RNA and various other charged molecules to inhibit the clearance of chromatin. We provide evidence for receptor-mediated clearance that can be inhibited by DNA. We also examine the effect of *in vivo* cleavage of cell surface proteoglycans by Heparinase I and provide evidence for participation of cell surface proteoglycans in clearance of chromatin. Treatment of mice with liposome-encapsulated Clodronate (dichloromethylene bisphosphonate) to deplete Kupffer cells and splenic macrophages dramatically decreased the rate of chromatin clearance. Our findings suggest that chromatin clearance is mediated by charge interactions with cell surface heparan sulphate proteoglycans (HSPG) and by specific cell surface receptors. Kupffer cells are required for the clearance process.

MATERIALS AND METHODS

Mice

C57Bl/10 J female mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6–8 weeks of age and kept at the animal facility at the VA Medical Center (Albuquerque, NM). Mice were used between 2 and 10 weeks after arrival. For each experiment animals were age-matched. The mice did not have antibodies to common rodent viruses.

Reagents

Tyramine, cellobiose, heparin, heparan sulphate, colominic acid, Heparinase I and carrier-free RNA were from Sigma (St Louis, MO). The colominic acid (poly 2,8 N-acetyl-neuraminic acid) had a molecular weight of 10 000. RNA preparations were analysed by agarose gel electrophoresis prior to use *in vivo* to verify stability of the preparations during storage. The stability of RNA *in vivo* was determined by measuring the rate of clearance of ^{125}I-labelled RNA. RNA was cleared relatively rapidly but by 5 min post-injection 28% of the injected dose remained in the circulation and TCA-precipitable.

Preparation of H1-stripped chromatin

H1-stripped chromatin was made exactly as described [10] using calf thymus (Pel Freeze Biologicals, Rogers, AK) as the starting material. Briefly, nuclei were isolated [11], and treated with micrococcal nuclease (Worthington Biochemicals Inc., Freehold, NJ) at 37°C as described [10]. The nuclei were lysed in low ionic strength buffer, centrifuged, and the supernatant brought to 0·55 M NaCl. This preparation was passed over a Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) column to separate H1 and non-histone proteins from the core histone–DNA complex, yielding long, soluble, H1-stripped chromatin. Fractions from the gel filtration column with nucleosome repeats of < 5 were pooled and used for clearance studies. SDS–PAGE and agarose gel electrophoresis of the pooled fractions showed protein bands corresponding to the four core histones and DNA bands in a 200-bp ladder. The H1-stripped chromatin was dialysed into 10 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7·4 and stored at −20°C in 50% glycerol. Calf thymus DNA was purchased from Calbiochem (La Jolla, CA) and further purified by digestion with proteinase K, extraction with phenol, and digestion with S1 nuclease [12]. Single-stranded DNA was made by placing dsDNA in a boiling water bath for 15 min and cooling rapidly on ice.

Radiolabelling of H1-stripped chromatin and RNA

H1-stripped chromatin was radiolabelled to a specific activity of 1–2 μCi/μg with ^{125}I-Na using 1,3,4,6-tetrachloro-3α6α-diphenylglycouril (IODO-GEN; Pierce, Rockford, IL). Briefly, 100 μg of H1-stripped chromatin in 100 μl PBS were added to a glass reaction tube precoated with 10 μg IODO-GEN. Carrier-free ^{125}I-Na (250 μCi) (ICN, Irvine, CA; 100 mCi/ml) was added to the chromatin, mixed gently, and incubated 15 min at room temperature. The reaction was terminated by the removal of the chromatin from the reaction tube. The H1-stripped chromatin was brought up to a volume of 1 ml and dialysed against PBS to remove free ^{125}I. The distribution of the radiolabel on the chromatin was determined after separating DNA from histones in 0·4 N H_2SO_4 (1 h at 21°C) and centrifugation (20 min at 10 000 ***g***) to pellet the DNA. Approximately 94% of the label was associated with histones for H1-stripped chromatin and 90·1% for tyramine cellobiose (TC)–chromatin. Autoradiography of these histones separated by SDS–PAGE showed labelling of H4 > H3 > H2B » H2A. RNA was radiolabelled with ^{125}I-Na using chloramine T as described [13].

Preparation of TC–chromatin

TC was prepared and radiolabelled according to the method of Pittman *et al*. [14]. H1-stripped chromatin was conjugated to TC using cyanuric chloride.

Analysis of clearance of chromatin from the circulation of mice

Mice were injected in one retroorbital sinus with 2 μg of ^{125}I-labelled H1-stripped chromatin in 200 μl of normal saline at time 0. Approximately 30 μl of blood were taken from the contralateral retroorbital sinus at the times indicated from 1 to 60 min. The exact amount of blood was determined by weighing. Fractions insoluble and soluble in 10% TCA were separated and counted in a gamma counter. For inhibition studies, mice were injected in one retroorbital sinus with 30 U Heparinase I 15 min before the injection of chromatin, or 100 μg heparin, heparan sulphate, or colominic acid 5 min before chromatin injection. DNA preparations were injected 2 min and RNA 30 s before chromatin injection. In some cases, dsDNA and heparin were injected after the chromatin, as indicated in the text. All calculations for radioactivity remaining in the circulation were based on the amount of chromatin present at 1 min post-injection. All experiments used at least three mice per group. Results are expressed as means ± s.d. The clearance rates of chromatin were measured by non-linear regression analysis using GraphPad Prism V 2.0 software from GraphPad Software Inc. (San Diego, CA). Comparison of curves for statistical differences was also performed by GraphPad Prism software using two-way ANOVA. Clearance was fitted to a one-phase or in some cases a two-phase exponential decay to maximize the goodness of fit. For organ localization the mice were killed at 60 min except for the TC–chromatin-injected mice, which were killed 24 h after injection of the TC–chromatin to allow for complete clearance of trapped radioactivity associated with blood in organs.

In vivo *depletion of Kupffer cells and splenic macrophages*

Liposomes were prepared containing dichloromethylene bisphosphonate (Clodronate) according to published procedures [15].

Clodronate was provided by Boehringer-Mannheim (Mannheim, Germany). Mice were injected with 0·2 ml of a 25% suspension (v/v) of liposomes 24–48 h prior to their use for clearance experiments. The depletion of Kupffer cells and splenic macrophages was verified by injecting mice with 0·2 ml of a 1:10 dilution of India Ink (Higgins, Newark, NJ) in PBS. The mice were killed after 3 h and the liver and spleen fixed and processed for haematoxylin and eosin staining. The slides were examined for cells taking up carbon particles. Clodronate treatment resulted in virtually complete elimination of carbon particle uptake in the liver and >95% depletion of the cells taking up carbon particles in the spleen.

Isolation of mouse liver cells

Mice were anaesthetized with Nembutal and a midline incision of the peritoneal cavity was used to expose the liver. The portal vein was cannulated and after cutting the inferior vena cava the liver was perfused with 500 ml of Hanks' balanced salt solution (HBSS) containing 10 mM HEPES buffer and 0·5 mM EGTA over 30 min. The liver was then perfused with 0·06% collagenase B (Boehringer-Mannheim) and 0·001% DNase I (Sigma) in perfusion buffer containing 1·3 mM $CaCl_2$ and 1 mM $MgCl_2$ and allowed to digest for 20 min. The liver was then removed and teased with forceps to produce a cell suspension. The cells were passed through a Dacron mesh screen to remove connective tissue elements and debris. They were then resuspended in ice-cold medium (Dulbecco's PBS with 1·8% glucose, 10 mM HEPES, 1 mM $MgCl_2$ and 0·2% bovine serum albumin (BSA)) and centrifuged three times at 50 ***g*** to pellet the hepatocyte fraction. The hepatocyte fraction was >95% pure by morphology after staining with Diff-Quik (Sigma) and by staining with diaminobenzidine for endogenous peroxidase. The supernatant from the first centrifugation of the liver cell suspension was washed and then taken as the non-parenchymal cell fraction. Contamination by parenchymal cells was always <5%.

RESULTS

Characterization of the H1-stripped chromatin preparation

Chromatin was prepared from calf thymus as described in Materials and Methods and analysed by SDS–PAGE for histone integrity and by agarose gel electrophoresis. The results of this analysis are shown in Fig. 1. The preparation contained approximately equal amounts of di- and mono-nucleosomes with about one half the number of trimers and half again the number of tetramers. The pentameric fraction was very small.

Inhibition of chromatin clearance by DNA

Previous studies had suggested that H1-stripped chromatin and core particles are cleared by a saturable process. To determine whether previously described receptors for DNA might be responsible for chromatin clearance, we tested the ability of DNA and RNA to inhibit clearance of H1-stripped chromatin. As seen in Fig. 2, both single-stranded and double-stranded DNA decreased the rate of chromatin clearance. DNA inhibition was dose-dependent, with partial inhibition at 5 μg and near complete inhibition at 50 μg or greater. The injection of 240 μg of RNA 30 s prior to injection of chromatin decreased clearance to a similar extent as 5 μg DNA.

Inhibition of chromatin clearance by heparin, heparan sulphate and Heparinase I treatment

Because in previous studies succinylated albumin interfered with the clearance of core particles [9], we decided to test the effect of other charged molecules on the clearance rate of H1-stripped chromatin. Injection of heparan sulphate or heparin inhibited clearance; however, colominic acid (a polymer of N-acetyl-neuraminic acid) did not (Fig. 3). It is thus unlikely that the interaction between chromatin and sites in the liver is simply charge-mediated.

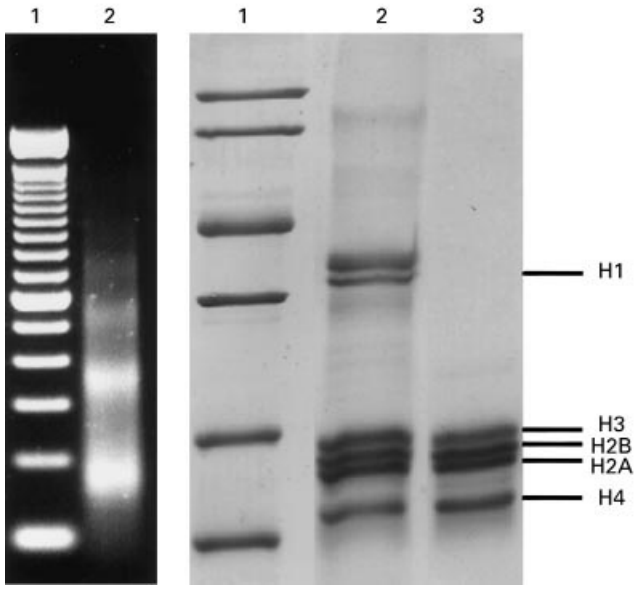

Fig. 1. Analysis of the H1-stripped chromatin used in the clearance studies. H1-stripped chromatin was prepared from calf thymus as described in Materials and Methods. The preparation was subjected to agarose gel electrophoresis (left panel) and SDS–PAGE (right panel). Left panel: Lane 1, 100-bp ladder; lane 2, H1-stripped chromatin. Right panel: Lane 1, mol. wt standards of 116·3, 97·4, 66·3, 55·4, 36·5 and 31·0 kD; lane 2, purified histones; lane 3, H1-stripped chromatin preparation.

Charged molecules have the potential to interact with the cell surface matrix. To test whether HSPG might be involved in the clearance of H1-stripped chromatin, mice were injected intravenously with 30 U of Heparinase I 15 min prior to chromatin injection. Heparinase I is specific for heparin but is effective in removing HSPG from hepatic cells due to the presence of significant heparin-like sequence in their cell surface proteoglycans [16]. Previous studies have demonstrated that Heparinase I can release up to 50% of the liver cell surface proteoglycans *in vivo* [17]. Heparinase treatment led to a marked decrease in the rate of chromatin clearance (Fig. 3). At 30 min, the percentage of TCA-insoluble ct/min remaining in circulation was 48·7 ± 6·9% for the heparinase group *versus* 29·7 ± 4·8% for the control group ($P = 0·01$). Since the liver is responsible for the vast majority of clearance of chromatin from circulation, the cleavage of hepatic HSPG by heparinase is the most likely explanation for these effects. It could not be determined whether the loss of HSPG from the cell surface caused a depletion of cell surface binding sites or if the cleaved HSPG chains could compete with chromatin to block binding.

Degradation of chromatin is not required for clearance

To determine if removal of chromatin from the circulation was associated with its degradation, the radioactivity in the TCA-soluble and -insoluble forms was examined As seen in Fig. 4a and as shown above, mice treated with DNA had a much decreased

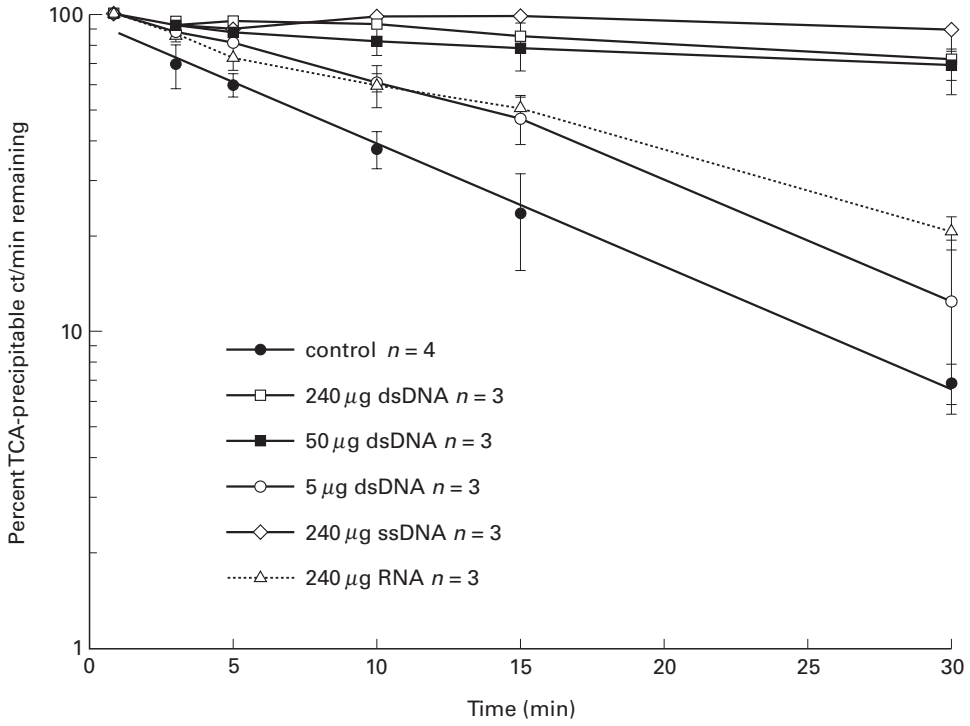

Fig. 2. Clearance of chromatin is blocked by dsDNA and by ssDNA. Mice were injected with 240, 50 or 5 μg of dsDNA or with 240 μg of ssDNA 2 min before injection of 2 μg of H1-stripped chromatin or with 240 μg of RNA 30 s prior to injection of chromatin. The results are expressed as the percentage of the injected radioactivity remaining in a TCA-precipitable form. The mean and s.d. are shown for three to four mice in each group. For all inhibitors shown the curves were significantly different from the control with $P < 0{\cdot}0001$.

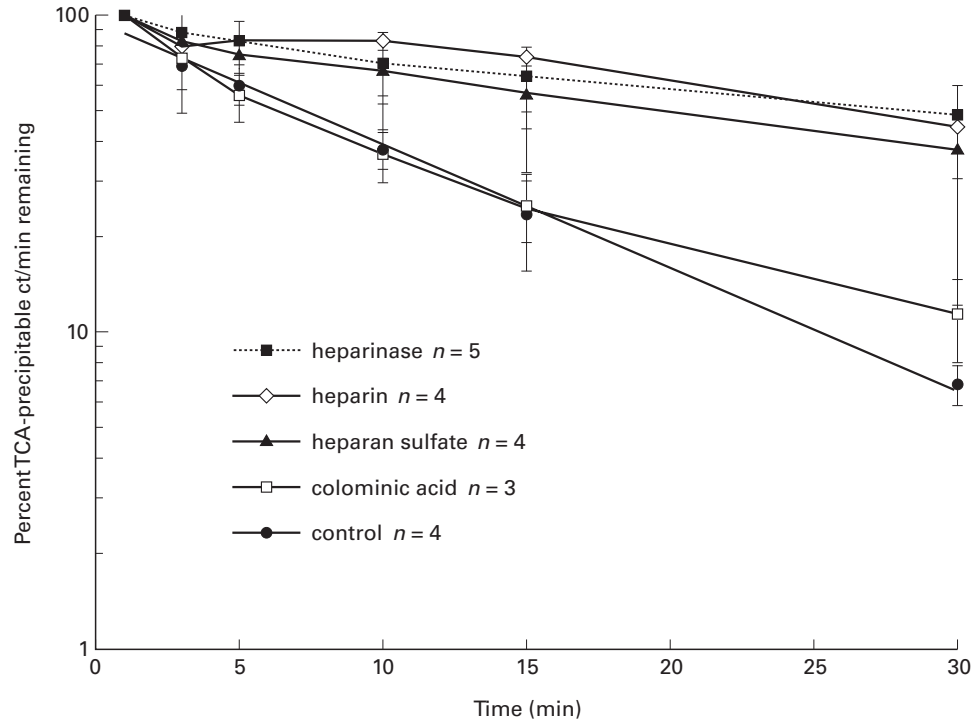

Fig. 3. Clearance of chromatin is blocked by heparin, by heparan sulphate, and by treatment with Heparinase I. Mice were injected with 100 μg colominic acid, 100 μg heparin or 100 μg heparan sulphate 5 min before injection of 2 μg H1-stripped chromatin or with 30 U Heparinase I 15 min prior to injection of chromatin. The results are expressed as the percentage of the injected radioactivity remaining in a TCA-precipitable form. The mean and s.d. are shown for three to five mice in each group. The clearance kinetics for Heparinase I, heparin and heparan sulphate all differ from the control ($P < 0{\cdot}001$). The clearance kinetics of the colominic acid-treatment group is not statistically different from the control.

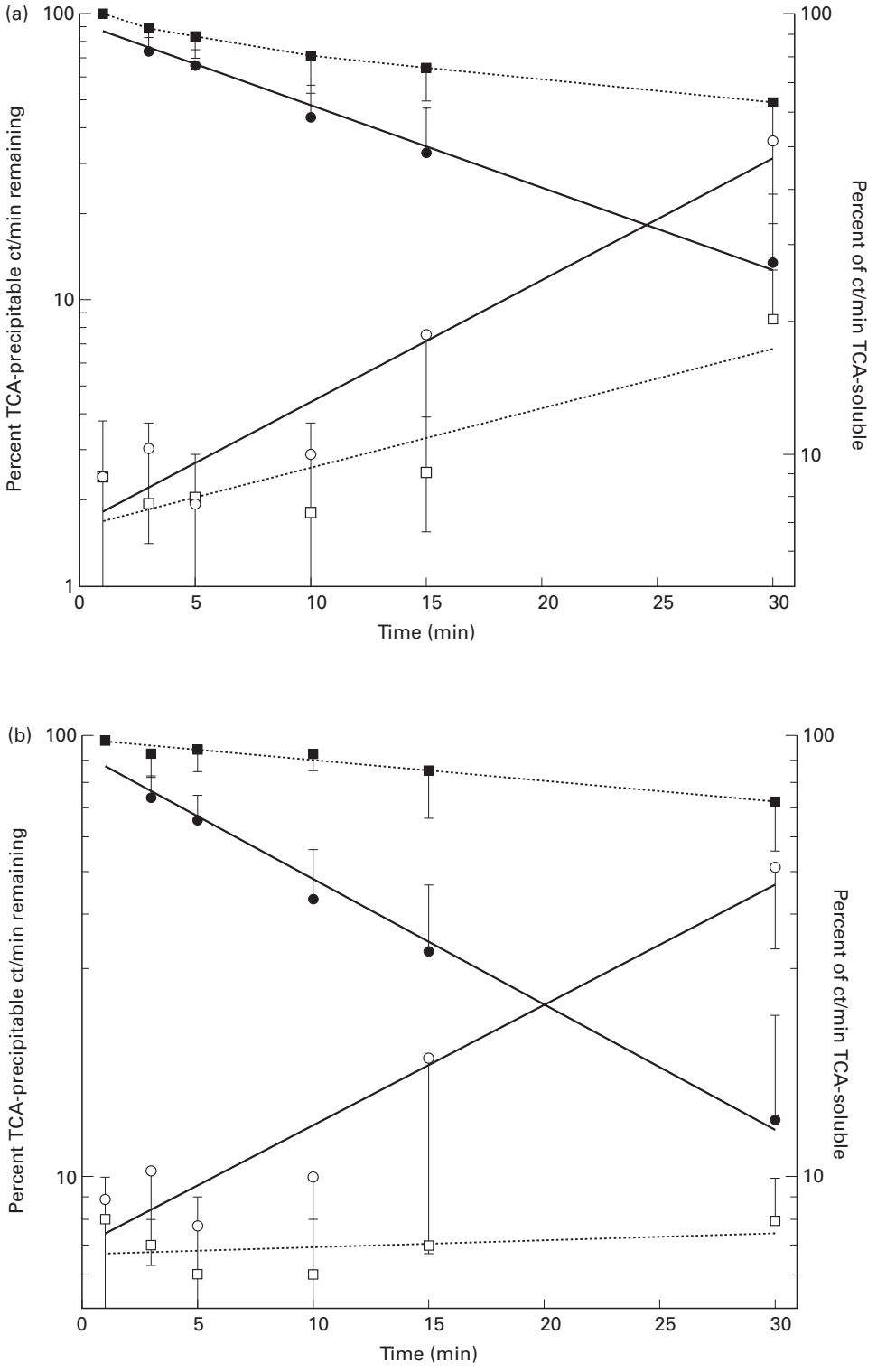

Fig. 4. Clearance of chromatin is not dependent on its degradation. Mice were injected with 2 μg of chromatin at time 0. (The mice are the same mice used in Figs 2 and 3 for the control, DNA and heparinase treatments.) (a) Mice received 240 μg of dsDNA 2 min prior to chromatin. ●, Mean % remaining in controls; ■, mean % remaining with heparinase; ○, mean % TCA-soluble in controls; □, mean % TCA-soluble with heparinase. (b) Mice were treated with Heparinase I 15 min prior to injection of chromatin. ●, Mean % remaining in controls; ■, mean % remaining with dsDNA block; ○, mean % TCA-soluble in controls; □, mean % TCA-soluble with dsDNA block. Results are expressed as the percentage of TCA-precipitable radioactivity remaining (closed symbols) and as the percentage of injected radioactivity remaining that is TCA-soluble (open symbols). The mean and s.d. are shown.

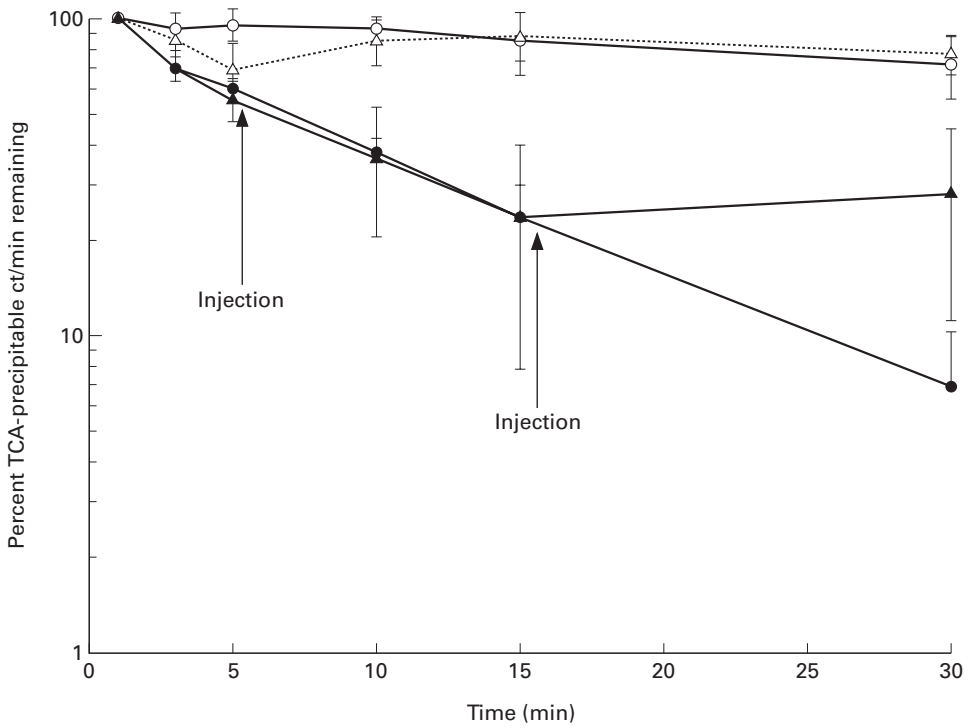

Fig. 5. Chromatin is released from cleared sites by dsDNA but not by heparin. Mice were injected intravenously with 2 μg of radiolabelled chromatin at time 0. Mice injected with 240 μg dsDNA either 5 min prior to chromatin, 5·5 min after chromatin or 15·5 min after chromatin (indicated by the arrows) are compared with control mice. The mean and s.d. are shown for three mice in each group. ●, Control ($n = 4$); ○, dsDNA preinject ($n = 3$); △, dsDNA 5·5 min post ($n = 3$); ▲, dsDNA 15·5 min post ($n = 3$).

rate of clearance. In addition, the circulating radiolabelled chromatin of the DNA-treated mice remained precipitable by TCA. In contrast, >50% of the circulating chromatin in the control mice was TCA-soluble by 30 min. The mechanism of this cleavage of chromatin is unknown. However, it is possible that this cleavage is mediated by a membrane protease and perhaps a nuclease as well that requires binding to the cell by a charge-mediated interaction. This effect is not specifically related to DNA treatment, as decreased degradation was also seen when clearance was inhibited using Heparinase I (Fig. 4b).

Release of chromatin from sites of clearance by dsDNA

Comparison of the clearance and degradation curves in Fig. 4 indicates that there is a delay in time between the removal of chromatin from the bloodstream and its release as a soluble product. To determine if the cleared chromatin was rapidly internalized or remained cell surface-associated during this time, the effect of injecting dsDNA at 5·5 min or 15·5 min after the injection of chromatin was tested. As seen in Fig. 5, 5·5 min after clearance was initiated virtually all of the cleared chromatin could be released from the bound sites and reappeared in the circulation in a TCA-insoluble form. At 15·5 min after the injection of chromatin, only a small percentage of the chromatin was released from clearance sites and reappeared in the circulation. In contrast, injection of heparin 5·5 min after chromatin released only a small percentage of the radioactivity, suggesting a less avid interaction of heparin with the binding molecule than dsDNA (data not shown). These results suggest that by 5·5 min chromatin was interacting more strongly with specific DNA-binding molecules than with HSPG. Thus, chromatin was cleared rapidly yet, for a short period, remained susceptible to competition by DNA. Degradation of chromatin probably takes place after internalization, as the releasable chromatin remains TCA insoluble. However, it should be noted that the fate of the DNA was not specifically determined since the chromatin was labelled on histones.

Organ localization of cleared chromatin

The fate of the cleared chromatin was examined by measuring radioactivity in the organs. Ten minutes after injection, 98% of the injected dose was recovered with 57% in the liver, 28% remaining in the blood, 9% in excretory organs, and <2% in other organs. Thus the liver was the predominant site of clearance. To confirm

Table 1. Organ distribution of cleared chromatin

	Form of chromatin injected	
Organ	Chromatin (%)	TC–chromatin (%)
Liver	90.3 (29)	93.0 (49)
Spleen	3.3 (1)	2.0 (1·1)
Kidneys	4.0 (1)	4.5 (2·4)
Heart	0.7 (<1)	0.1 (<1)
Lungs	1.7 (<1)	0.1 (<1)

Percentage of total radioactivity recovered (% of injected dose in parentheses) in organs. Results are the means of three animals per group. The tyramine cellobiose (TC)–chromatin was group was killed at 24 h after injection.

that the organ counts were reflective of the clearance site of chromatin rather than digestion fragments, H1-stripped chromatin was conjugated to ^{125}I-labelled TC. TC is a non-degradable disaccharide that is catabolized slowly by lysosomes and trapped at the site of initial clearance of the attached molecule. The mice were killed 24 h after injection of the TC–chromatin to allow for complete clearance of trapped blood in organs. The clearance rate of TC–chromatin was very similar to that of chromatin that had not been coupled. In addition, the clearance pattern of chromatin with respect to organ distribution was comparable (Table 1). Thus, the clearance pattern obtained for chromatin is indicative of the initial site of chromatin clearance rather than reflecting digested chromatin and secondary binding sites.

Elimination of chromatin clearance by depletion of macrophages in vivo

Because Kupffer cells have been implicated in the clearance of many molecules from the circulation, the effect of depletion of Kupffer cells from the animal was measured. Depletion of Kupffer cells and splenic macrophages was accomplished by injection of liposomes containing Clodronate into mice 24–48 h before the clearance procedure. Clodronate depletes Kupffer cells and splenic macrophages by a 'suicide' process rather than saturating their phagocytic capacity. This technique has been shown to deplete macrophages (Kupffer cells) effectively from the liver and from the spleen [15,18]. The depletion of macrophages in the mice in this study was confirmed by examining tissue sections of the liver and spleen for India Ink deposition in mice treated with Clodronate liposomes and controls. The injection of Clodronate liposomes had a profound effect on the clearance rate of the H1-stripped chromatin, with a change in half-life from 15 min to 146 min (Fig. 6). It should be noted that the initial 20% of the chromatin cleared was not affected by Clodronate treatment, suggesting a separate mechanism for initial clearance, possibly by interaction with hepatocytes.

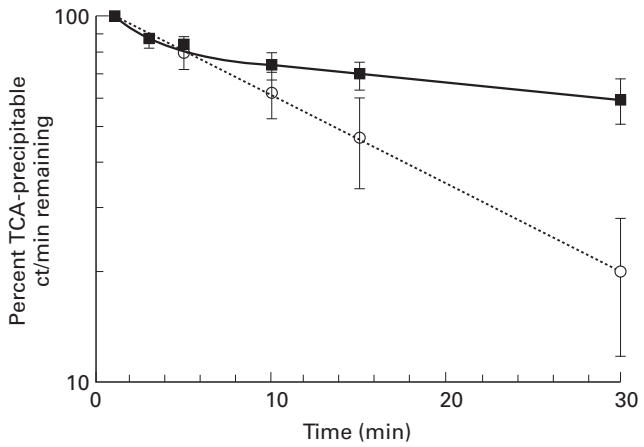

Fig. 6. Clearance of chromatin in mice is eliminated by pretreatment with Clodronate-containing liposomes. Mice were injected with Clodronate-containing liposomes 24–48 h prior to the clearance experiment. Mice were then injected with 2 μg of H1-stripped chromatin at time 0 and blood was collected at the times indicated. The results are expressed as the percentage of the injected radioactivity remaining in a TCA-precipitable form. The mean and s.d. are shown for three mice in each group. The clearance kinetics of the Clodronate group (■, $n = 3$) is statistically different from the control (○, $n = 3$) ($P < 0{\cdot}0001$).

Table 2. Recovery of injected chromatin in parenchymal and non-parenchymal cell fractions

Experiment	Parenchymal cell (ct/min)/10^6 cells	Non-parenchymal cell (ct/min)/10^6 cells	Ratio* (ct/min)/10^6 cells
1	97	18 280	188:1
2	575	102 371	178:1
3	379	82 740	218:1

* Ratio of ct/min recovered in the non-parenchymal cell fraction to the ct/min recovered in the parenchymal cell fraction.

Recovery of radioactivity in liver parenchymal cell and non-parenchymal cell fractions

To determine whether cleared chromatin was associated with the parenchymal cell fraction, animals were injected with radiolabelled chromatin and then killed after 10 min. The livers were perfused to eliminate loosely associated radioactivity and then injected with collagenase and the liver cells isolated. The parenchymal cells were separated by a series of low speed centrifugations. The non-parenchymal fraction was taken from the supernatant of the first low speed separation of the hepatic parenchymal cells. It can be seen from Table 2 that, consistent with the ability of Clodronate to inhibit clearance, the non-parenchymal cell fraction was much more efficient in taking up chromatin on a per cell basis.

DISCUSSION

The clearance of chromatin from the circulation is of central importance to the pathogenesis of SLE, which is characterized by the deposition of pathogenic immune complexes in the glomeruli of affected individuals. Although earlier studies had clearly defined the mechanisms involved in clearance of DNA [1–4,19], relatively little is known about the clearance of chromatin. As chromatin is likely to be the form of DNA present in the circulation [6], the study of its clearance may be more relevant to the pathogenesis of SLE.

Previous studies examined the fate of core particles and of H1-stripped chromatin cleared from the circulation [7–9]. Our studies revealed that clearance might vary markedly between strains of mice with high and low levels of SAP [8]. SAP is an acute-phase serum protein and a member of the pentraxin family of proteins. SAP is synthesized in the liver and binds to chromatin. Mice with low levels of SAP, such as C57Bl/10, have rapid rates of clearance whereas BALB/c mice, which have high levels of SAP, have slow clearance rates. Induction of the acute-phase response or injection of CRP, another member of the pentraxin family that also binds chromatin and is the major acute-phase protein in humans, or SAP slows the rate of clearance in C57Bl/10 mice. Therefore, we chose to examine further the clearance of chromatin in the C57Bl/10 mice.

Our previous studies and those of others indicated that chromatin is cleared primarily by the liver. However, it was not determined whether chromatin clearance was a receptor-mediated process. We found that chromatin clearance was saturable (data not shown), and could be blocked by pretreatment of mice with DNA. The ability of low concentrations of DNA to inhibit clearance of chromatin suggests that the same receptors might be responsible for clearing chromatin and DNA.

The clearance rate of chromatin was also markedly decreased after treatment of mice with Heparinase I, which removes cell surface HSPG from the liver. It is known that HSPG are involved in the clearance of many macromolecules, protozoa and bacteria. These studies do not specifically determine whether HSPG are present on Kupffer cells. However, ongoing *in vitro* studies suggest that cell surface HSPG are not involved in uptake of chromatin by macrophages. It is possible that HSPG mediate the observed binding of chromatin to hepatic parenchymal cells, but not the ultimate clearance by Kupffer cells. A role for HSPG has been demonstrated for binding of DNA-containing immune complexes to the glomerular basement membrane (GBM) [20]. These authors showed that binding of MoAb to GBM required binding to nucleosomal antigens and was most dependent on the presence of histones.

Interactions between molecules cleared via HSPG may be either sequence-specific with respect to the ligand cleared or mediated by charge interactions alone. An example of the former is the clearance of malaria sporozoites in which interaction between the sporozoite and the cell has been related to the presence of a short, basically charged sequence of amino acids in the circumsporozoite protein that covers the cell surface of the parasite [21,22]. Specific interactions with unique sugar sequences have been defined. Our data demonstrate that a variety of charged molecules, including sulphated and non-sulphated species, were able to abrogate the clearance, suggesting that the clearance of chromatin is more likely to involve a charge-mediated interaction. However, colominic acid and RNA, which are both negatively charged macromolecules, were not effective inhibitors of chromatin clearance. The ability of DNA, but not heparin, to release cleared chromatin back into the circulation at early time points suggests that there may be a charge-mediated interaction with HSPG and a more specific interaction with DNA-binding proteins.

It is of interest that the chromatin in the circulation remained 90% TCA-insoluble if clearance was delayed. However, a large increase in TCA-soluble counts was seen following its interaction with fixed sites, primarily in the liver. Degradation and resistance to release by DNA occurred coincidentally, suggesting that cleavage was predominantly intracellular. This would argue against a major role for enzymes in the circulation in the degradation of chromatin. It seems likely that the interaction with the liver and specifically Kupffer cells leads to degradation and subsequent release of chromatin fragments. This hypothesis is further supported by the ability of liposomes containing Clodronate to block clearance and degradation of chromatin. Treatment with liposomes containing Clodronate eliminates Kupffer cells and splenic macrophages. It should be noted that the clearance of chromatin during the first 10 min was not blocked by Clodronate and this accounted for 20% of the injected dose of chromatin. This may account for the association of chromatin with the parenchymal cell fraction in the studies of separated liver cell fractions. Clearly the non-parenchymal cell fraction is more efficient on a per cell basis, however.

It is yet to be determined if a specific receptor for chromatin exists on these cells, and if it is involved in clearance. Studies have suggested that a specific receptor for DNA is present on hepatic cells [23]. Similar to the findings presented here, this receptor is localized on non-parenchymal cells. The identity of this receptor remains to be determined. Bennett *et al*. have described a receptor for DNA on circulating leucocytes, including 98% of monocytic cells [24]. Similar to our findings, receptor binding of labelled DNA was not inhibited by RNA but was inhibited by DNA and by heparin. More recently, a receptor for nucleosomes was reported on the surface of fibroblasts [25]. However, the relationship of this receptor to the previously described DNA receptor is also unknown. It is unclear whether chromatin would also use this mechanism for clearance. However, DNA was the most effective inhibitor of the clearance of chromatin. DNA was also able to release bound chromatin back into the circulation and to alter the site of clearance of chromatin. In addition, 240 μg of RNA had very little effect on clearance rates of chromatin, whereas 50 μg of DNA markedly slowed chromatin clearance. Injection of DNA 60 min before injection of chromatin also inhibited clearance. These findings suggest that inhibition of chromatin clearance by DNA does not result from inhibition of a charge interaction with HSPG, but is due to a more specific inhibition of chromatin binding to sites that also bind to DNA.

In conclusion, chromatin is cleared by Kupffer cells in the liver and macrophages in the spleen. This process is inhibited by treatment with heparinase, heparin or heparan sulphate, suggesting a role for cell surface proteoglycans in clearance. The most effective inhibitor of clearance was DNA, which completely blocked chromatin clearance and was capable of releasing bound chromatin. These data suggest that chromatin may bind to a specific receptor, which along with cell surface proteoglycans participates in clearance.

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REFERENCES

1 Tsumita T, Iwanaga M. Fate of injected deoxyribonucleic acid in mice. Nature 1963; **198**:1088–9.
2 Chused TM, Steinberg AD, Talal N. The clearance and localization of nucleic acids by New Zealand and normal mice. Clin Exp Immunol 1972; **12**:465–76.
3 Emlen W, Mannik M. Kinetics and mechanisms for removal of circulating single-stranded DNA in mice. J Exp Med 1978; **147**:684–99.
4 Emlen W, Mannik M. Clearance of circulating DNA–anti-DNA immune complexes in mice. J Exp Med 1982; **155**:1210–5.
5 Emlen W, Mannik M. Effect of DNA size and strandedness on the in vivo clearance and organ localization of DNA. Clin Exp Immunol 1984; **56**:185–92.
6 Rumore PM, Steinman CR. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. J Clin Invest 1990; **86**:69–74.
7 Du Clos TW, Zlock L, Hicks PS *et al*. Decreased autoantibody levels and enhanced survival of (NZB X NZW) F_1 mice treated with C-reactive protein. Clin Immunol Immunopathol 1994; **70**:22–27.
8 Burlingame RW, Volzer MA, Harris J *et al*. The effect of acute phase proteins on clearance of chromatin from the circulation of normal mice. J Immunol 1996; **156**:4783–8.
9 Gauthier VJ, Tyler LN, Mannik M. Blood clearance kinetics and liver uptake of mononucleosomes in mice. J Immunol 1996; **156**:1151–6.
10 Lutter OC. Kinetic analysis of deoxyribonuclease I cleavage in the nucleosome core: evidence for a DNA superhelix. J Mol Biol 1978; **124**:391–420.
11 Godfrey JE, Baxevanis AD, Moudrianakis EN. Spectropolarimetric

analysis of the core histone octamer and its subunits. Biochem 1990; **29**:965–72.
12 Rubin RL. Enzyme-linked immunosorbent assay for anti-DNA and antihistone antibodies. In: Rose NR, Friedman H, Fahey JL, eds. Manual of clinical laboratory immunology. Washington: ASM, 1986:744–9.
13 Shaspohnikov JD, Zerov YP, Ratovitski EA *et al. In vitro* RNA iodination with the aid of chloramine T. Anal Biochem 1976; **75**:234–40.
14 Pittman RC, Carew TE, Glass CK *et al*. A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation. Biochem J 1983; **212**:791–800.
15 van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994; **174**:83–93.
16 Lyon M, Deakin JA, Gallagher JT. Liver heparan sulfate structure: a novel molecular design. J Biol Chem 1994; **269**:11208–15.
17 Ji Z-S, Sanan DA, Mahley RW. Intravenous heparinase inhibits remnant lipoprotein clearance from the plasma and uptake by the liver: in vivo role of heparan sulfate proteoglycans. J Lipid Res 1995; **36**:583–92.
18 van Rooijen N, van Nieuwmegen R. Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. Cell Tissue Res 1984; **238**:355–8.
19 Emlen W, Mannik M. Effect of preformed immune complexes on the clearance and tissue localization of single-stranded DNA in mice. Clin Exp Immunol 1980; **40**:264–72.
20 Kramers C, Hylkema MN, Van Bruggen MCJ *et al*. Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo. J Clin Invest 1994; **94**:568–77.
21 Cerami C, Frevert U, Sinnis P *et al*. Rapid clearance of malaria circumsporozoite protein (CS) by hepatocytes. J Exp Med 1994; **179**:695–701.
22 Frevert U, Sinnis P, Cerami C *et al*. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. J Exp Med 1993; **177**:1287–98.
23 Emlen W, Rifai A, Magilvay D *et al*. Hepatic binding of DNA is mediated by a receptor on nonparenchymal cells. Am J Pathol 1988; **133**:54–60.
24 Bennett RM, Gabor GT, Merritt MM. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. J Clin Invest 1985; **76**:2182–90.
25 Koutouzov S, Cabrespines A, Amoura Z *et al*. Binding of nucleosomes to a cell surface receptor: redistribution and endocytosis in the presence of lupus antibodies. Eur J Immunol 1996; **26**:472–86.